

Themed Section: Endothelin

REVIEW

The role of the paracrine/autocrine mediator endothelin-1 in regulation of cardiac contractility and growth

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Endothelin-1 (ET-1) is a critical autocrine and paracrine regulator of cardiac physiology and pathology. Produced locally within the myocardium in response to diverse mechanical and neurohormonal stimuli, ET-1 acutely modulates cardiac contractility. During pathological cardiovascular conditions such as ischaemia, left ventricular hypertrophy and heart failure, myocyte expression and activity of the entire ET-1 system is enhanced, allowing the peptide to both initiate and maintain maladaptive cellular responses. Both the acute and chronic effects of ET-1 are dependent on the activation of intracellular signalling pathways, regulated by the inositol-trisphosphate and diacylglycerol produced upon activation of the ET_A receptor. Subsequent stimulation of protein kinases C and D, calmodulin-dependent kinase II, calcineurin and MAPKs modifies the systolic calcium transient, myofibril function and the activity of transcription factors that coordinate cellular remodelling. The precise nature of the cellular response to ET-1 is governed by the timing, localization and context of such signals, allowing the peptide to regulate both cardiomyocyte physiology and instigate disease.

LINKED ARTICLES

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Abbreviations

AngII, angiotensin II; CICR, calcium-induced-calcium release; Cn, calcineurin; ECC, excitation-contraction-coupling; IICR, IP₃-induced calcium release; IP₃R, inositol triphosphate receptor; MyBP-C, myosin binding protein C; RyR, ryanodine receptor; TnI, troponin I

Introduction

Endothelin (ET)-1, ET-2 and ET-3 are a family of cyclic 21 amino acid peptides. The peptides are encoded within three separate yet highly conserved genes located on chromosomes 6, 1 and 20 in humans respectively (Inoue *et al.*, 1989). ET-1 is the most well characterized member of this family. The peptide was initially isolated from the culture supernatant of porcine aortic endothelial cells and identified as a protease-sensitive vasoconstrictor. It remains the most potent vasoactive substance identified to date (Yanagisawa *et al.*, 1988), performing an important function in the regulation of vascular smooth muscle tone. ET-1 also mediates effects on other cell types. Here, we discuss the role of ET-1 in the heart. We will focus on the actions of ET-1 on cardiac muscle and

describe how the ET system contributes to cardiac regulation and autoregulation during health and disease.

ET-1 in the heart

Throughout life, ET-1 plays key roles in many aspects of cardiac physiology and pathology. It is involved in controlling aortic arch formation during development (Kurihara *et al.*, 1995), is required for cardiomyocyte survival and prevents myocyte loss during ageing (Zhao *et al.*, 2006). In the adult, ET-1 modulates coronary blood flow by regulation of vascular tone. Furthermore, by acting on its receptors expressed by atrial and ventricular myocytes, the peptide modulates cardiac muscle function directly (Hirata *et al.*,

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1989). The effects of ET-1 on the heart are observed over distinct and wide timescales. Acutely (within minutes), ET-1 modulates muscle contractility and induces arrhythmias (Baltogiannis *et al.*, 2005), whereas over longer time scales (days to weeks), the peptide induces cardiomyocyte growth. The long-term effects of ET-1 are associated with maladaptive hypertrophic remodelling of the heart (Yorikane *et al.*, 1993) and its progression to failure (Francis *et al.*, 1990). Hence, ET-1 is considered to contribute to both the aetiology and pathology of these conditions. Indeed, the circulating plasma level of ET-1 is positively correlated with severity of cardiac disease and thus is a reliable prognostic indicator of future heart failure (Selvais *et al.*, 2000; Zolk *et al.*, 2002).

Paracrine/autocrine ET-1 in regulation of cardiac function

Further contributing to its significant role in modulating cardiomyocyte function, ET-1 is synthesized and secreted by the myocytes (Nunez *et al.*, 1990; Firth and Ratcliffe, 1992; Suzuki *et al.*, 1993), fibroblasts (Fujisaki *et al.*, 1995) and endothelial cells (Kedzierski and Yanagisawa, 2001) of the heart. The presence of the machinery for both the production and detection of ET-1 within the myocardium generates a local autocrine and paracrine ET-1 system, providing a tightly coupled and regulated means of control (Pikkarainen *et al.*, 2006; Higazi *et al.*, 2009). Supporting this notion, ET-1 production/secretion is signal-responsive and contributes to both normal adaptive physiological responses and to the induction and progression of pathology. During physiology, myocyte-secreted ET-1 is reported to contribute to stretch-induced enhancement of contractility, which can occur during periods of increased workload (Pikkarainen *et al.*, 2006). In this context, ET-1 secretion/production is either directly stimulated or induced in response to AngII, which is also secreted by the stretched myocyte (Sadoshima *et al.*, 1993; Yamazaki *et al.*, 1996; Pikkarainen *et al.*, 2006; Cingolani *et al.*, 2011). As a result, contractility is enhanced to accommodate for the change in cardiac demand. This mechanism may explain the slow force response or Anrep effect observed in stretched atrial preparations (Kockskämper *et al.*, 2008a). Indeed, many of the cardiac effects of AngII are abrogated by ET receptor inhibition (Ito *et al.*, 1993). Should the initiating stimulus and thus ET-1 secretion persist, hypertrophy may result (Yamazaki *et al.*, 1996). The acute effect of ET-1 is therefore a prequel to its longer term effect on cardiac remodelling (Cingolani *et al.*, 2011). In aged individuals, the plasma concentration of ET-1 is elevated (Maeda *et al.*, 2003), hence the importance of ET-1-induced cardiovascular effects increases with time. Furthermore, cardiomyocyte ET-1 generation is increased following ischaemia (Serneri *et al.*, 2000), during the cardiac hypertrophic response (Yorikane *et al.*, 1993; Zolk *et al.*, 2002; Mayyas *et al.*, 2010) and as a result of heart failure (Francis *et al.*, 1990). This increased ET-1 contributes to pathologies such as arrhythmia and the initiation and development of cardiac remodelling, the incidence of which increases with age (Lakatta and Levy, 2003b). Increased expression of ET-1 has been observed at both the mRNA level of pre-pro-ET-1 and the protein level of the mature peptide

itself (Arai *et al.*, 1995; Kobayashi *et al.*, 1999; Zhao *et al.*, 2006), suggesting that increased transcription contributes to enhanced ET-1 production. This is accompanied by increased activity of the processing mechanisms that produce the mature peptide. Expression and activity of endothelin converting enzyme 1a (ECE1a) is up-regulated in human failing hearts and animal models of congestive heart failure (Ergul *et al.*, 2000a; 2000b), expediting the processing of big-ET-1 into mature ET-1. In conjunction with the increased level of ET-1 peptide, expression of the ET_A receptor by ventricular myocytes is enhanced during disease (Arai *et al.*, 1995; Pieske *et al.*, 1999; Baltogiannis *et al.*, 2005; Harzheim *et al.*, 2010). Thus, under disease-associated conditions, mechanisms of ET-1 production and detection are up-regulated in a myocyte-centric manner, providing an entirely local mechanism for the pathological effects of ET-1.

ET receptors

ET-1 elicits its effects by association with one or both of its receptor isoforms, the ET_A and ET_B receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Webb, 1991; Yorikane *et al.*, 1993; Alexander *et al.*, 2011). Both receptor isoforms are classical GPCRs comprising 7 α -helical transmembrane spanning domains, an extracellular N-terminal domain and intracellular C-terminal domain. The intracellular C-terminal domain and loops between the transmembrane segments interact with heterotrimeric G-proteins composed of G α , β and γ subunits. Activation of these receptors stimulates multiple intracellular signalling pathways and mediates diverse cellular effects (Ishikawa *et al.*, 1988a; 1988b; Moravec *et al.*, 1989). The relative abundance of ET receptors varies across the chambers and regions of the normal healthy heart, with the number of ET-1 binding sites in atrial cells far exceeding those found in ventricular cells (Molenaar *et al.*, 1993). Functionally, the ET_A and ET_B receptors exhibit several key differences. Although the affinity of both ET receptors for ET is subnanomolar, the ET_A receptor exhibits a higher affinity for ET-1 over ET-2 or ET-3 (Hosoda *et al.*, 1991), whilst the ET_B receptor binds non-selectively to each of the three ET isoforms (Sakurai *et al.*, 1990). The downstream signals activated following engagement of the ET_A receptor and ET_B receptor also differ. Both receptors can couple to multiple G-proteins but the particular G-protein repertoire that each isoform engages may vary, governing the pattern of downstream pathways activated. The ET_A receptor interacts with G $\alpha_{q/11}$ (Aramori and Nakaniishi, 1992), G α_s (Eguchi *et al.*, 1993b; Yamazaki *et al.*, 1996) and G α_{12} activating polyphosphoinositide hydrolysis and production of inositol-(1,4,5)-triphosphate (IP₃), diacylglycerol (DAG) and cAMP (Griendling *et al.*, 1989; Takuwa *et al.*, 1990; Serneri *et al.*, 2000). The ET_B receptor also couples to G $\alpha_{q/11}$. However, in contrast to the ET_A receptor, it preferentially interacts with G $\alpha_{i/o}$ and G α_{13} (Eguchi *et al.*, 1993b; Gohla *et al.*, 1999). Consequently, activation of the ET_B receptor is associated with inhibition of cAMP accumulation as well as stimulation of polyphosphoinositide hydrolysis (Eguchi *et al.*, 1993a). Although the heart expresses both receptor isoforms, in cardiomyocytes the ET_A receptor predominates accounting for greater than 80% of ET binding sites (Allen *et al.*, 2003).

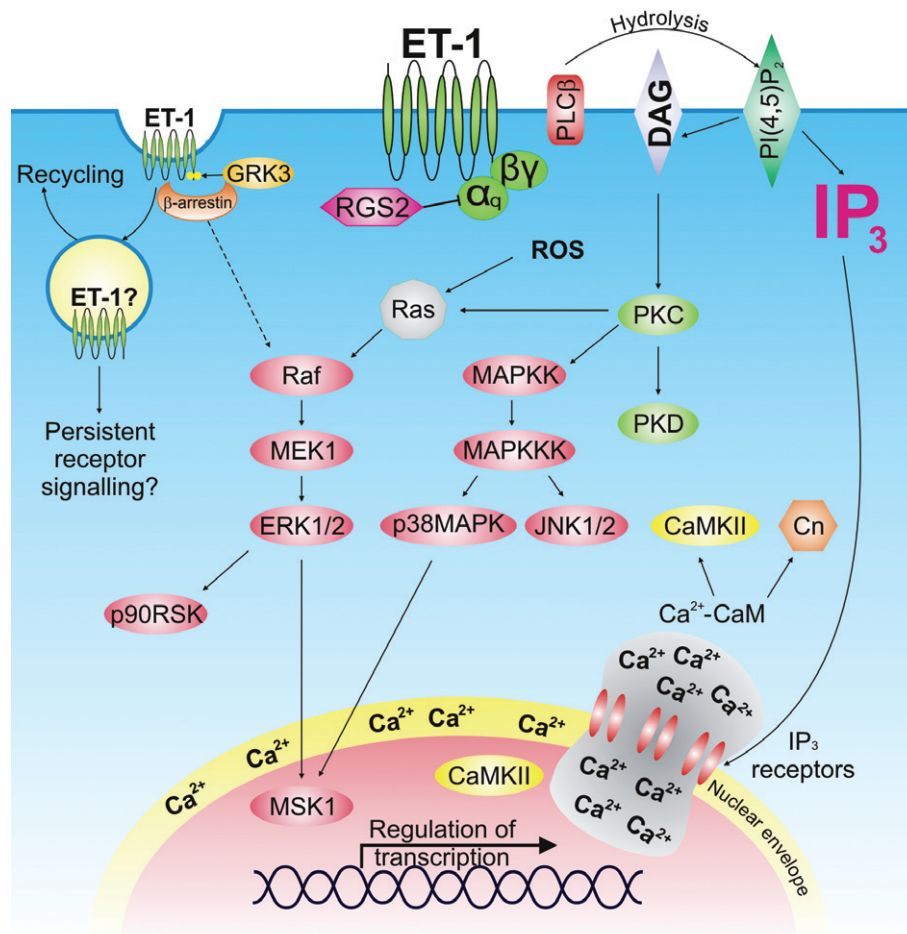


Figure 1

Signalling pathways activated downstream of the ET_A receptor in cardiomyocytes. ET-1 binds to the $G\alpha_q$ -coupled ET_A receptor inducing production of IP_3 and DAG. These second messengers activate protein kinase cascades, ROS production and IP_3 -induced calcium release. Following ET-1 binding, the ET-1- ET_A receptor complex is internalized in a GRK3- β -arrestin dependent manner. β -arrestin has been postulated to induce activation of MAPK cascades.

The most well established mechanisms of ET_A receptor-dependent signalling in cardiomyocytes concern those regulated downstream of $G\alpha_{q/11}$. Inhibition of $G\alpha_{q/11}$ prevents activation of ET-1 dependent signalling pathways in rat adult ventricular myocytes (AVM), whilst reducing the activity of either $G\alpha_{12/13}$ or $G\alpha_{i/o}$ does not produce comparable effects (Snabaitis *et al.*, 2005). Furthermore, ET-1-dependent signalling is enhanced by knockdown of the GTPase activating protein regulator of G-protein signalling 2 (RGS2), which negatively regulates GTP-bound $G\alpha_{q/11}$ by enhancing its GTP hydrolysing properties (Zhang *et al.*, 2006). The downstream effects of $G\alpha_{q/11}$ activation are mediated through activation of PLC enzymes, which catalyse the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PI-4,5- P_2) to produce membrane-associated DAG and soluble IP_3 (Wu *et al.*, 1992). Cardiomyocytes express multiple PLC isoforms, with PLC β_1 and B_3 showing $G\alpha_{q/11}$ -dependent activity. ET-1 stimulation of cultured neonatal rat ventricular myocytes (NRVM) leads to phosphoinositide hydrolysis within seconds (Clerk and Sugden, 1997). The rapid increase in IP_3 and DAG activates multifarious downstream signalling pathways, including

those regulated by calcium, small G-proteins and kinase cascades (Figure 1). In addition, ET-1 stimulates nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (De Giusti *et al.*, 2008) and production of reactive oxygen species (ROS) (Cheng *et al.*, 1999). These pathways perform important roles in regulation of cardiomyocyte contractility, pacemaking and remodelling.

Terminating ET-1 signalling

In order to limit the effects of ET-1 on the heart, signalling from ET receptors must be tightly regulated. The ET_A receptor forms a very high affinity interaction with ET-1 and the rate of disassociation of ligand/receptor complex is slow; hence, this process is not straightforward (Hilal-Dandan *et al.*, 1997). Indeed, the resistance of the ET-1/ ET_A receptor complex to acid or exposure to inhibitor has led to the proposal that ET-1/ ET_A receptor binding is quasi-irreversible, possibly as a result of covalent binding of ET-1 to the receptor. Thus, it is likely that the receptor is internalized in a ligand-bound state

and that internalization of this complex, rather than ET-1 dissociation, determines the duration of the ET-1 response. Indeed, an association of ET-1 with the ET_A receptor within the endosome for up to 2 h has been reported (Chun *et al.*, 1994; 1995). It is unclear whether ET-1 continues to signal via the ET_A receptor when internalized in this manner but this hypothesis could explain certain of the observed effects of ET-1 stimulation. Co-localization of internalized ET-1-ET_A receptor with caveolar domains may aid interaction with G-proteins, enabling ET-1-ET_A receptor signalling to persist (Chun *et al.*, 1994). Furthermore, ET-1-ET_A receptor complex internalization is GRK (G-protein receptor kinase)- β -arrestin dependent (Freedman *et al.*, 1997). GRKs 2, 3 and 5 are expressed in cardiac myocytes (Vinge *et al.*, 2001) with GRK2 activity and expression being up-regulated in heart failure – an adaptation that limits β -adrenergic signalling (Ping *et al.*, 1997). In AVMs, GRK3 targets the ET_A receptor and inhibition of GRK3 in AVM enhances ET-1 induced signalling, suggesting that this kinase is important for attenuation of the ET-1 response (Vinge *et al.*, 2007). Following GRK-dependent phosphorylation, recruitment of β -arrestins to activated receptors targets them for internalization. However, β -arrestin is also implicated in the activation of the MAPK cascade (Lefkowitz and Shenoy, 2005). Given these potential mechanisms of alternative and persistent ET-1-induced signalling, it appears that a simple model of ET-1-GPCR binding and agonist-induced desensitization cannot fully account for the cellular effects of ET-1.

Interestingly, internalized ET_A receptor and ET_B receptor are targeted to different intracellular compartments after stimulation with ET-1. The ET_A receptor co-localizes with the pericentriolar recycling compartment and can be recycled to the plasmalemma so that cellular ET-1 sensitivity is maintained. In contrast, ET_B receptor are targeted to lysosomes and degraded after agonist-induced internalization. The lysosomal fate of the internalized ET-1-ET_B receptor complex provides a mechanism for clearance of the peptide (Attinà *et al.*, 2005). Exemplifying this, deletion of the ET_B receptor in mice impairs ET-1 clearance and selective pharmacological blockade of this isoform increases the circulating concentration of ET-1 (Gariépy *et al.*, 2000; Kelland *et al.*, 2010). The vasculature of the lung provides the major site of clearance of ET-1 from plasma (Dupuis *et al.*, 1996), with approximately 80% of the peptide being removed from the bloodstream by the ET_B receptors expressed by pulmonary endothelial cells. Efficient removal of the peptide via this mechanism generates a half-life for ET-1 in the circulation of less than 5 min and maintains the plasma concentration of the peptide in healthy individuals at approximately 1 pM (Kedzierski and Yanagisawa, 2001), significantly below its activity threshold. However, local ET-1 concentration at sites of ET-1 production and release is expected to be much higher, raising the level of the peptide to that which can modulate cardiac function.

ET-1 in cardiac pacemaking and contractility

Cardiac contraction during systole is initiated by an action potential generated following automatic depolarization of

the pacemaking SA node. From the SA node, the action potential propagates across the atria and induces contraction. Following a pause at the atrio-ventricular node, the action potential is rapidly conducted to the apex of the heart by specialized Purkinje fibres. Subsequent spreading of the action potential throughout the ventricles induces contraction and thus expels blood into the aorta. At the molecular level, the mechanism that links membrane excitation and myocyte contraction is termed excitation-contraction coupling (ECC) (Figure 2) (Bers, 2002; Fearnley *et al.*, 2011). This process is initiated by the depolarization-induced opening of L-type voltage-gated calcium channels (also known as dihydropyridine receptors), generating the I_{Ca,L} current. Calcium entry through these channels generates a much larger release of calcium from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR2), in a process termed 'calcium-induced-calcium-release' (CICR) (Fabiato, 1983; Roderick *et al.*, 2003). This calcium binds to the contractile apparatus and induces cellular shortening. To terminate systole and allow relaxation, calcium is extruded from the cell or re-sequestered in the SR. Synchronous release of calcium throughout the ventricular myocyte is achieved as a consequence of the highly specialized subcellular structure of the cell. The sarcolemma is extensively invaginated to form the t-tubular network; hence, depolarizing action potentials can spread into the depths of the cell and induce coordinated cellular contraction. Furthermore, the L-type calcium channel and RyR2 are co-localized within specialized structures known as the cardiac dyads, facilitating CICR.

ET-1 has an established role in the regulation of cardiac contractility, in particular as a positive inotrope (Ishikawa *et al.*, 1988b; Moravec *et al.*, 1989; Shah *et al.*, 1989). Other effects upon the heart include negative inotropy, arrhythmogenesis, decreased relaxation (lusitropy) and chronotropy (Ishikawa *et al.*, 1988a; Li *et al.*, 2005). Despite these well-described aspects of ET-1 in cardiac biology, controversy remains regarding the effects of ET on cardiac function, its mechanism(s) of action and the specific molecular targets involved. A number of factors contribute to the apparent diversity of responses reported, although variable expression levels of ET receptors account for many of the discrepancies. For example, responses are different between species and vary with age. ET-1 does not exert an inotropic effect in adult mice and dogs, which have low levels of ET receptor expression (Takanashi and Endoh, 1991; Nishimaru *et al.*, 2007). However, in neonatal mice, where ET receptors are more abundant, inotropic responses to ET-1 are observed (Nagasaka *et al.*, 2003). Moreover, the origin of the cardiac preparation studied is also a significant determinant of the response (Moravec *et al.*, 1989). This is not surprising given that the distribution and abundance of ET receptor isoforms varies across the chambers of the heart (Molenaar *et al.*, 1993; Russell and Davenport, 1996). Further diversity is provided by the experimental preparation – intact heart, chamber muscle strips or isolated cells.

Due to greater receptor abundance in the atria, the effects of ET-1 are most well established in this chamber (Ishikawa *et al.*, 1988b; Vigne *et al.*, 1989; Molenaar *et al.*, 1993). The atrial response to ET-1 (e.g. in rat, rabbit and guinea pig) is characterized by an initial negative inotropic phase (first 1–2 min), followed by a slowly developing and persistent positive

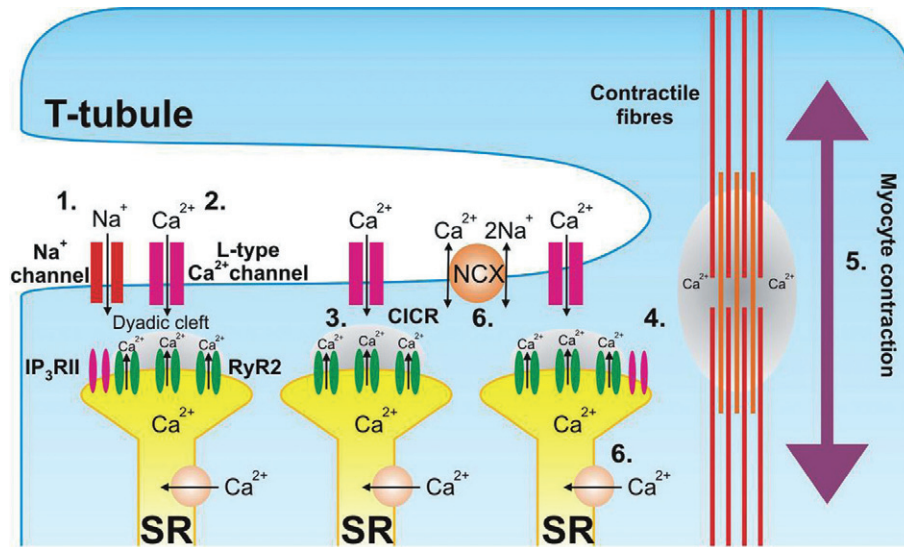


Figure 2

Excitation–contraction coupling in the cardiomyocyte. Opening of voltage-gated Na^+ -channels induces cellular depolarization (1). L-type voltage-gated calcium channels are activated in response to depolarization and mediate calcium entry (2). Increased calcium concentration within the cardiac dyad activates the closely opposed RyR2 and induces calcium-induced-calcium release (3), raising cytosolic calcium concentration. Calcium diffuses to the contractile apparatus (4), binds to the contractile filaments and stimulates contraction (5). Calcium is re-sequestered in the sarcoplasmic reticulum or extruded from the cell by NCX, returning cytosolic calcium concentration to resting levels (6). Colocalization of $\text{IP}_3\text{R1}$ within the cardiac dyad facilitates calcium-induced calcium release under stimulated conditions.

inotropic phase (Takanashi and Endoh, 1991; Bootman *et al.*, 2007). Although cAMP independent, the positive inotropic effect of ET-1 in the atria can reach levels comparable to that induced by the catecholamines (Vigne *et al.*, 1989). The cardiac ventricle also exhibits a positive inotropic response to ET-1, albeit at a substantially reduced magnitude (Takanashi and Endoh, 1991; Proven *et al.*, 2006) and unlike the situation in the atria, a negative inotropic response is rarely observed (Proven *et al.*, 2006). Notably, ET-1 also affects heart rate by modulating the physiology of the SA node and Purkinje fibres (Ishikawa *et al.*, 1988a). The combined effect of ET-1-induced positive inotropy and chronotropy increases cardiac output under conditions of stress, contributing to the adaptive response of the heart. However, these actions of ET-1 also have deleterious consequences, promoting arrhythmogenic, action potential-independent calcium transients (Baltogiannis *et al.*, 2005; Li *et al.*, 2005; Mayyas *et al.*, 2010).

Similarly to other cardiotoxic agonists, ET-1 changes cardiac contractility by modulating components of the ECC machinery (Figure 3). These include alterations in the dynamics and amplitude of systolic calcium transients (Bootman *et al.*, 2007; Kocksamper *et al.*, 2008), changes in myofilament sensitivity to calcium (Yang *et al.*, 1999; Westfall *et al.*, 2005; Cuello *et al.*, 2007a) or altered frequency of stimulation (Ishikawa *et al.*, 1988a).

ET-1 regulation of NCX activity

ET-1 effects systolic calcium predominantly via modulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) (Ballard and Schaffer, 1996; Yang *et al.*, 1999) and L-type channel activity (Ono *et al.*,

1994). The primary function of NCX is calcium clearance from the cytosol during diastole, accounting for up to 20% of the calcium extruded, a contribution that increases further under disease conditions (Bers, 2002; 2006). NCX uses the electrochemical gradient for Na^+ between the extracellular space and the cytosol, created by the $\text{Na}^+\text{K}^+\text{ATPase}$, to exchange 3 Na^+ for every calcium ion extruded from the cell (Philipson and Nicoll, 2000). Where intracellular calcium is increased independently of an action potential, NCX-mediated Na^+ entry may cause sufficient depolarization to cross the threshold for action potential generation (Gilbert *et al.*, 1991). As well as this forward mode of activity, NCX can also operate in reverse, inducing calcium influx (Leblanc and Hume, 1990). This may be precipitated by raised intracellular Na^+ concentration under conditions of membrane depolarization, for example during the initial phase of the action potential (Shattock and Bers, 1989). As a result of calcium influx at this point, calcium transients are increased in amplitude and contractility enhanced (Meyer *et al.*, 1996; Yang *et al.*, 1999). ET-1 regulates NCX either by engaging signalling mediators activated directly downstream of the ET_A receptor such as PKC (Ballard and Schaffer, 1996; Zhang and Hancox, 2009), or by increasing activity of the sodium-hydrogen exchanger (NHE) (Cingolani *et al.*, 2011). The NHE is the major mechanism by which intracellular pH is regulated in cardiac myocytes. It extrudes one H^+ ion from the cell in exchange for 1 Na^+ (Cingolani and Ennis, 2007), exploiting the Na^+ gradient created by the $\text{Na}^+\text{K}^+\text{ATPase}$. The resulting increase in intracellular Na^+ activates NCX in reverse mode, increasing $[\text{Ca}^{2+}]_i$ and producing an inotropic effect (Alvarez *et al.*, 1999). H^+ extrusion by NHE would also be expected to modulate contractility via cytosolic alkalinization, which

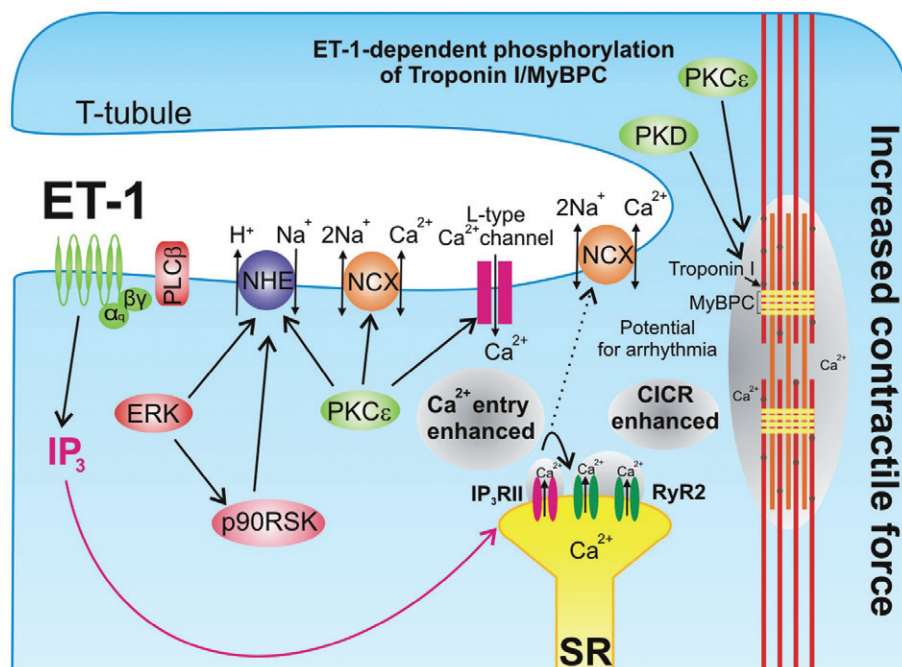


Figure 3

ET-1 regulates ventricular myocyte contractility. Downstream of the ET_A receptor in the ventricular myocyte, PLCβ, PKC, PKD, p90 RSK and ERK are activated. Protein-kinase-dependent phosphorylation of NHE1 increases intracellular Na⁺ concentration and depolarizes the cell. Subsequent activation of NCX in reverse mode allows calcium entry to be enhanced. When combined with PKC-dependent amplification of calcium entry through the L-type calcium channel, the stimulus for CICR is increased. IP₃-dependent calcium release from the SR sensitizes the closely opposed RyR2, hence facilitating CICR. The resulting elevated cytosolic calcium concentration and PKC/PKD-dependent phosphorylation of myofilament proteins allows the myocyte to produce a larger force of contraction.

would influence myofilament calcium sensitivity. Despite support for this hypothesis in earlier studies, it is now clear that the small changes in intracellular [H], produced by NHE are compensated for by the bicarbonate anion transporter; providing bicarbonate is present in the extracellular medium (Alvarez *et al.*, 1999). Therefore, this mechanism is unlikely to contribute to the ET-1 response. ET-1 increases NHE activity via kinases such as PKC, ERK1/2 and p90 RSK (Bianchini *et al.*, 1997; Fliegel and Karmazyn, 2004; Maekawa *et al.*, 2006; Cuello *et al.*, 2007b; Snabaitis *et al.*, 2008). Although ERK1/2 and therefore p90 RSK may both be activated via canonical GPCR signalling, NADPH oxidase activation and ROS formation are necessary for these kinases to phosphorylate NHE in response to ET-1 (Cingolani *et al.*, 2011).

ET-1 regulation of L-type calcium current

L-type calcium channel activity is subject to extensive regulation by GPCRs, which elicit their effects through PKC and PKA dependent phosphorylation (Puri *et al.*, 1997; Kamp and Hell, 2000). Phosphorylation of the pore-forming α_{1C} and accessory α_{1S} and β_{2A} subunits of the channel has been detected biochemically (Puri *et al.*, 1997). Although intensively studied, there is a lack of consensus regarding modulation of L-type calcium current (I_{Ca,L}) by ET-1, with evidence

for both a positive (Lauer *et al.*, 1992; He *et al.*, 2000) and negative effect (Ono *et al.*, 1994; 2001; Cheng *et al.*, 1995). A biphasic effect of ET-1 has been reported in rabbit ventricular myocytes, consisting of a reduction followed by an enhancement of I_{Ca,L} (Watanabe and Endoh, 1999). In these cells the ET-1 stimulated augmentation of I_{Ca,L} approached a level similar to that induced by β-adrenoreceptor stimulation. However, the mechanisms by which ET-1 and sympathetic stimuli affect I_{Ca,L} are not shared. When ET-1 and sympathetic stimuli occur concurrently, ET-1 exerts an anti-adrenergic effect through a pertussis toxin-sensitive pathway (i.e. G_{i/o} mediated). This suppresses the enhancement of I_{Ca,L} by β-agonists and prevents their associated inotropic (Boixel *et al.*, 2001; Munzel *et al.*, 2005) and arrhythmic responses (Kolettis *et al.*, 2008). In addition to the inter-species variation in the ET-1 response, the lack of a consistent effect of ET-1 has also been ascribed to the methods used for recording I_{Ca,L} (He *et al.*, 2000). In order to mitigate this, the perforated patch technique has been used to minimize intracellular dialysis and loss of signalling mediators. Using this approach, a consistent PKC-dependent positive regulation of I_{Ca,L} by ET-1 has been reported (Kamp and Hell, 2000). In order for such a mechanism to operate efficiently, co-localization of the main players is required to facilitate interaction of the DAG generated by phosphoinositide hydrolysis with its targets (Robu *et al.*, 2003). The importance of DAG localization is illustrated by the contrasting effects of bath-applied and intracellular-released DiC8, a DAG analogue (He *et al.*,

2000). Bath application of DiC8 induces a PKC-independent reduction in $I_{Ca,L}$ whereas intracellular photoactivation of caged DiC8 mimics the PKC-dependent enhancement of $I_{Ca,L}$ observed with ET-1 application (He *et al.*, 2000). Although PKC phosphorylates and activates L-type channels directly, the effects of PKC activation could also be transduced by calcium/calmodulin-dependent protein kinase II (Komukai *et al.*, 2010). Furthermore, ET receptors, PLC β and PKC ϵ are all localized to the sarcolemma of the t-tubule in ventricular myocytes, bringing them into close proximity with L-type calcium channels (Robu *et al.*, 2003). Interestingly, in disease situations where t-tubules are lost, ET-1 no longer elicits a positive inotropic effect (Chung *et al.*, 2008; Smyrniak *et al.*, 2010).

ET-1 stimulated IP₃-induced calcium release and cardiac contractility

As well as DAG, ET-1-dependent phosphoinositide hydrolysis in the myocytes and conduction system of the heart generates the important signalling effector IP₃ (Chen *et al.*, 2004; Remus *et al.*, 2006; Hirose *et al.*, 2008; Kockskämper *et al.*, 2008b; Ju *et al.*, 2011). Due to the relatively small increase in IP₃ and low expression level of IP₃ receptors (IP₃Rs) in heart preparations relative to other tissues, the role, if any for IP₃ in modulating intracellular [Ca²⁺] and contractility was for a long time unclear (Wu *et al.*, 2006; Higazi *et al.*, 2009). Furthermore, since expression of IP₃Rs is 50–100 fold lower than that of the RyR (Moschella and Marks, 1993; Perez *et al.*, 1997; Lipp *et al.*, 2000), it was difficult to discern what additional function IP₃Rs could perform. This contrasts with the prominent role for IP₃Rs in the development and physiology of the embryonic heart. Indeed, mice in which the type 1 and type 2 IP₃R isoforms (IP₃RI and IP₃RII) are subject to germ-line deletion are not viable and exhibit defects in cardiac development (Uchida *et al.*, 2010). Moreover, IP₃ stimulated calcium signals play a key role in pacemaker activity of the embryonic heart (Kapur and Banach, 2007). Evidence has recently accumulated which indicates that despite low expression of IP₃Rs, the IP₃ signalling pathway also plays a significant role in the physiology and pathophysiology of the adult heart, contributing to many of the effects of ET-1 and other G α_q coupled receptors on cardiac function (Wu *et al.*, 2006; Kockskämper *et al.*, 2008b; Higazi *et al.*, 2009; Nakayama *et al.*, 2010). As would be expected, the contribution of IP₃ signalling to the action of ET-1 across the regions of the heart varies according to the level of expression and subcellular localization of the IP₃R. Similarly to ET_A receptors, IP₃Rs are most abundant in atrial myocytes and in nodal tissue (Lipp *et al.*, 2000; Ju *et al.*, 2011). The IP₃RII predominates in SA node, atrial and ventricular myocytes (Perez *et al.*, 1997; Lipp *et al.*, 2000; Ju *et al.*, 2011), whereas in Purkinje fibres, IP₃RI is most abundant (Gorza *et al.*, 1993).

Engagement of IP₃ signalling in the different cell types of the heart has varying consequences. In the SA node, ET-1 causes increases in firing rate, calcium transient amplitude, diastolic calcium levels and the frequency of elementary calcium release events (Ju *et al.*, 2011). The recapitulation of these effects by exposure of nodal cells to cell-permeant IP₃

and the decreased firing rate and loss of ET-1 responses in nodal cells from IP₃R knockout mice demonstrates the importance of ET-1/IP₃ signalling in this region of the heart (Ju *et al.*, 2011). Although ET-1 also elicits a positive chronotropic effect in guinea pig SAN (Ishikawa *et al.*, 1988a), rabbit SAN exhibits a negative chronotropic response to ET-1 (Ono *et al.*, 2001). The effect of ET-1 in these rabbit cells was attributed to modification of calcium and potassium currents. The role of IP₃ signalling in eliciting the effects of ET-1 upon the SAN in species other than mouse remains to be fully resolved. ET-1 also elicits spontaneous events in Purkinje fibres. Despite evidence for expression of functional IP₃Rs that contribute to spontaneous calcium release events in these cells (Gorza *et al.*, 1993; Wu and Tseng, 1993; Boyden *et al.*, 2004), it is not determined whether these IP₃Rs are engaged by ET-1 (Wu and Tseng, 1993). The IP₃R is however stimulated in Purkinje cells by IP₃ generated following activation of α -adrenergic GPCRs, resulting in wide long lasting calcium release events (Hirose *et al.*, 2008).

In atrial myocytes, IP₃ signalling contributes to the inotropic and pro-arrhythmic action of ET-1 (Li *et al.*, 2005), and IP₃-mediated calcium signals are localized to the sub-sarcolemmal and perinuclear regions (Mackenzie *et al.*, 2004; Zima *et al.*, 2007). Whilst perinuclear IP₃-mediated calcium signals have been proposed to control transcription (see later) (Wu *et al.*, 2006; Higazi *et al.*, 2009), IP₃-mediated calcium signals at the plasma membrane play a role in modulating atrial myocyte contractility and may underlie the enhanced automaticity of ET-1 stimulated myocytes (Mackenzie *et al.*, 2004). A similar pattern of IP₃R expression and activity is observed in cells of the SAN (Ju *et al.*, 2011). As a result of their lack of t-tubules, ECC is restricted to the peripheral sarcolemma of atrial myocytes (Ju *et al.*, 2011). Therefore, IP₃-mediated calcium signals exert a greater than anticipated effect on the physiology of an ET-1-stimulated atrial or SAN myocyte due to their co-localization with the ECC machinery in these cells. ET-1-stimulated IP₃-mediated calcium signals promote transition of the sub-sarcolemmal ring of calcium normally observed following depolarization of atrial myocytes to a global calcium elevation, enhancing ECC and recruitment of RyRs deeper in the myocyte cell volume (Mackenzie *et al.*, 2004). As a result, contractility is increased and blood propelled with greater force into the ventricles. Since the extent of ventricular stretch determines the contractile force produced by the ventricle – the Frank Starling relationship – greater atrial contraction brings about increased cardiac output. In addition to enhancing electrically evoked calcium transients, ET-1 application also increases the incidence of elementary calcium release events in atrial myocytes (Mackenzie *et al.*, 2001; Li *et al.*, 2005). These events contribute to an increase in diastolic calcium levels beneath the sarcolemma, which if sufficiently elevated, activates NCX-dependent inward Na⁺ flux and action potential generation. As a result, a potentially arrhythmogenic ectopic event is induced (Lipp *et al.*, 2000; Mackenzie *et al.*, 2002).

Functional interactions between IP₃Rs and RyRs are also observed in ventricular myocytes exposed to ET-1 (Proven *et al.*, 2006; Domeier *et al.*, 2008; Harzheim *et al.*, 2009). However due to the lower abundance of ET and IP₃ receptors in these cells (Lipp *et al.*, 2000; Domeier *et al.*, 2008), their interaction and influence upon myocyte physiology is not as

great. Most notably, IP₃ signalling does not make a prominent contribution to the inotropic action of ET-1 in the ventricular myocyte. This is not surprising given the limited effect of ET-1 upon calcium fluxes in these cells when compared with atrial myocytes (Proven *et al.*, 2006; Domeier *et al.*, 2008). Prolonged exposure of ventricular myocytes to ET-1 does however lead to the generation of ectopic calcium transients and IP₃-dependent extra-systolic contractions (Proven *et al.*, 2006) and IP₃-dependent calcium signals also promote sodium-calcium exchange (Gilbert *et al.*, 1991). Thus, similarly to the situation in atrial myocytes, ET-1 activated IP₃ signalling has the potential to precipitate ventricular arrhythmias.

As discussed earlier, both circulating and local cardiac ET-1 levels are increased with age (Maeda *et al.*, 2003) and during cardiac pathologies such as arrhythmia, infarction and hypertrophy (Yorikane *et al.*, 1993; Serneri *et al.*, 2000). Although this increase in ET-1 may initially support ECC, it also has deleterious consequences and is likely to exacerbate pathology and/or cause it to persist (Tuinenburg *et al.*, 1998; Baltogiannis *et al.*, 2005; Mayyas *et al.*, 2010). Furthermore, IP₃Rs are increased in their abundance during cardiac pathology (Moschella and Marks, 1993; Harzheim *et al.*, 2009; 2010). As a result, the effects of ET-1 on myocyte physiology are enhanced. Notably, the additional IP₃Rs in the hypertrophied heart are not homogeneously distributed throughout the cell volume but localized to the sub-sarcolemmal dyadic region, proximal to the RyRs that participate in ECC (Harzheim *et al.*, 2009). Thus, under conditions of increased IP₃, calcium emanating from these IP₃Rs stimulates calcium release from their neighbouring RyRs. The effects of this activity are twofold. First, IICR may promote ECC, thereby enhancing contractility. However, second, IICR may promote potentially arrhythmogenic ectopic calcium release events (Harzheim *et al.*, 2009). On balance, the enhancement of calcium release via RyRs by the additional IP₃Rs may outweigh the potential deleterious effects of ectopic calcium signals. During hypertrophy and the progression to failure, decreased coupling efficiency between L-type channels and RyRs arises as a result of increased width of the dyadic cleft (Song *et al.*, 2006; Xu *et al.*, 2007; Heinzel *et al.*, 2011). In this situation, calcium release through additional IP₃Rs could provide a mechanism to overcome this defect in ECC by bringing RyRs closer to their threshold for activation. This effect is analogous to the priming of RyRs by PKA phosphorylation following β -stimulation, which sensitizes the receptors to calcium influx via LTCC (Zhou *et al.*, 2009). In this way, increased IP₃R expression may be considered an adaptive response that sustains contractile function as the heart hypertrophies.

Effects of ET-1 upon the contractile apparatus

The relationship between the enhancement in $[Ca^{2+}]_i$ and contractility observed in cardiac preparations following exposure to ET is non-linear, indicating that the inotropic response does not solely result from enhancement of intracellular calcium levels. This non-linearity is particularly appar-

ent in ventricular preparations where, despite a significant increase in contraction, $[Ca^{2+}]_i$ is not substantially augmented (Wang *et al.*, 1991; Pieske *et al.*, 1997). This contrasts with the response to the catecholamines, which cause a robust increase in calcium transient amplitude and contractility. The mechanism by which the alteration in contraction is brought about by catecholamines is well defined and occurs primarily through PKA-dependent phosphorylation of contractile filament regulatory proteins, most notably the inhibitory subunit of troponin (TnI) and myosin binding protein C (MyBP-C) (Chen *et al.*, 2010). Phosphorylation of TnI and MyBP-C differentially contributes to regulation of contractility. Phosphorylation of TnI reduces calcium sensitivity of the myofilaments (Pi *et al.*, 2003), and phosphorylation of MyBP-C is responsible for enhanced cross-bridge cycling, thereby enhancing relaxation (Chen *et al.*, 2010). GPCRs that signal via G α_q such as the ET_A receptor elicit an opposing effect on myofilament action – generally increasing sensitivity to calcium and decreasing cross-bridge cycling (Pi *et al.*, 2003; Wang *et al.*, 2006). As such, their modulation of contractility is energetically less expensive. PKC, RSK and PKD have all been identified as ET-1-activated TnI and MyBP-C kinases (Cuello *et al.*, 2007a; 2011; Chen *et al.*, 2010).

Remodelling of the heart by ET-1

In addition to its diverse actions in the regulation of myocyte contractility, ET-1 has been consistently demonstrated to perform a major role in disease-associated remodelling of the heart (Russell and Molenaar, 2000; Sugden, 2003). Heart disease encompasses a spectrum of interrelated and progressive disorders, which include hypertension, coronary artery disease, arrhythmia and, most severely, heart failure (Diwan and Dorn, 2007). Pathological enlargement of the heart, termed maladaptive cardiac hypertrophy, is observed clinically in many of these conditions and can be induced as a result of increased arterial blood pressure, genetic mutations or following myocardial injury. Hypertrophic remodelling can take place within the left and/or right ventricles, depending on the nature of the initiating stimulus. It develops as a consequence of the increased size of individual ventricular myocytes, without an increase in myocyte number. When hypertrophy occurs as a consequence of persistent cardiovascular stress, an increase in heart size is a pathological and maladaptive response. As the condition progresses to heart failure, myocyte apoptosis/necrosis and myocardial fibrosis are observed, impairing cardiac contractility and reducing cardiac output. At the cellular level, hypertrophic myocytes show remodelling of gene expression profiles, altered intracellular calcium handling and reorganization of the contractile machinery. Reprogramming of cardiomyocyte gene expression profiles is responsible for many of the phenotypic changes observed in hypertrophic myocytes and is perceived to be critical for disease-associated impairment of myocyte function. Left ventricular hypertrophy produced by reprogramming under pathological conditions is one of the major risk factors for subsequent progression of patients to congestive heart failure and sudden death (Jessup and Brozena, 2003), prompting much investigation into the mechanism of action of pro-hypertrophic stimuli. Exposure of cultured car-

diomyocytes to ET-1 has been shown to recapitulate many of the cellular features of hypertrophic myocytes (Ito *et al.*, 1991). Furthermore, ET-1 stimulation of NRVM alters expression of hundreds of genes, in a manner mirroring the remodelling of gene expression observed during hypertrophy (Cullingford *et al.*, 2008). Consequently, these observations have promoted much investigation into the role of the ET-1 system in pathological remodelling.

As indicated earlier, cardiomyocyte ET-1 biosynthesis and secretion and ET receptor expression are increased in heart failure patients and animal models of cardiovascular conditions (Hasegawa *et al.*, 1996; Motte *et al.*, 2003; McMullen and Jennings, 2007). This remodelling of the ET system thereby provides a local mechanism for the pathological effects of ET-1. The importance of a local role for ET-1 in the induction of myocardial remodelling has been demonstrated by the effects observed upon infection of an organism with the intracellular protozoan *Trypanosoma cruzi*. Parasite infection leads to the development of Chagas disease (Coura and Viñas, 2010), which in its chronic form induces a cardiomyopathic phenotype, consisting of cardiomyocyte hypertrophy, myocardial fibrosis and, eventually, myocyte death. Together, these changes promote the development of heart failure (Coura and Borges-Pereira, 2010). Similar to other forms of hypertrophic remodelling, plasma ET-1 concentration is high in mice and humans infected with *T. cruzi* and mRNA levels of both ET-1 itself and the enzymes involved in its processing are increased under these conditions (Petkova *et al.*, 2000; Salomone *et al.*, 2001). When the ET-1 gene is specifically deleted in cardiomyocytes, the maladaptive effects of *T. cruzi* infection are reduced. Such a protective effect is not observed upon deletion of ET-1 in endothelial cells, suggesting a myocyte-specific ET-1-dependent origin for the cardiomyopathic effect of the disease (Tanowitz *et al.*, 2005).

Manipulation of the ET-1 system experimentally in murine models of hypertrophy and heart failure has provided valuable further insight into the role of the peptide in left ventricular remodelling. Cardio-specific conditional overexpression of ET-1 in mice induces a pathological hypertrophic phenotype, which can be attenuated by combined blockade of ET_A and ET_B receptors (Yang *et al.*, 2004), whilst cardio-specific deletion of the ET-1 gene inhibits the hypertrophic response to treatment with the T3 thyroid hormone (Shohet *et al.*, 2004). Infusion of the ET_A receptor antagonist, BQ123, inhibits the response to a pro-hypertrophic stimulus in adult rats (Ito *et al.*, 1994) and when administered to rats experiencing chronic heart failure, BQ123 impedes left ventricular remodelling and improves survival (Sakai *et al.*, 1996). These observations support the conclusion that ET-1 and the ET_A receptor have an important role in pathological remodelling of the heart.

Based on these findings, the signalling pathways activated downstream of ET-1 and ET_A receptor activation have been extensively studied within the context of the hypertrophic response. Overexpression of PLCβ_{1b}, a primary proximal target of the ET_A receptor, is sufficient to induce a hypertrophic response in NRVM (Filtz *et al.*, 2009). Moreover, by inducing calcium signals and activation of PKC, respectively, the IP₃ and DAG generated following ET-1 stimulated PLC-mediated phosphoinositide hydrolysis have pro-hypertrophic effects.

Role of IP₃-dependent calcium release in hypertrophy

The low baseline expression of the IP₃RII in ventricular myocytes (Perez *et al.*, 1997) has impeded investigation into its role in hypertrophic remodelling. Moreover, it has been unclear how IICR arising from the relatively few cardiomyocyte IP₃RII could be discriminated from the cyclical global release and reuptake of calcium that mediates cellular contractility. However, it has been recognized that IP₃RII expression increases during cardiovascular disease, with increased receptor expression being observed in human cardiomyopathic hearts and animal models of hypertrophic remodelling (Go *et al.*, 1995; Harzheim *et al.*, 2009; 2010; Nakayama *et al.*, 2010). As well as being expressed in the dyadic region, these receptors are also found in the perinuclear region. IP₃-dependent nuclear calcium signals occur in response to ET-1 and are required to elicit a hypertrophic response (Wu *et al.*, 2006; Higazi *et al.*, 2009). Furthermore, a mouse-model with cardio-specific overexpression of the IP₃RII demonstrated enhanced IP₃-induced calcium release (IICR) and mild baseline hypertrophy (Nakayama *et al.*, 2010). Together, these data suggest that IICR has a pro-hypertrophic effect on the myocardium and that nuclear IICR plays a key role in the hypertrophic response to ET-1. The observation that ET-1 elicits release of calcium specifically in the nuclear domain is significant as it would allow pro-hypertrophic IP₃-dependent calcium signals to be detected independently from the bulk calcium transients that elicit myocyte contractility. Furthermore, calcium elevation within the nucleus co-localizes the calcium signal with calcium-sensitive kinases and regulators of transcription, spatially coupling the stimulus with its effectors (Goonasekera and Molkentin, 2012). Such mechanisms are likely to be crucial for the remodelling of gene expression profiles observed in hypertrophy. Calcineurin-nuclear factor of activated T cells (Cn-NFAT) (Higazi *et al.*, 2009; Nakayama *et al.*, 2010; Rinne and Blatter, 2010) and CaMKII (Wu *et al.*, 2006), are examples of IICR-regulated proteins that can be localized within the nucleus. Significantly, both have important roles in the hypertrophic response.

Calcineurin (Cn) is a calcium-dependent heterotrimeric serine-threonine phosphatase (Clipstone and Crabtree, 1992; Fruman *et al.*, 1992). In response to increases in calcium concentration such as those elicited by ET-1-induced IP₃ generation, IP₃, the Cn autoinhibitory domain is displaced from the active site, stimulating Cn phosphatase activity. The major cellular target of activated Cn is a family of 4 Rel-homology domain containing transcription factors, NFATc1-4 (Jain *et al.*, 1993). Subcellular localization of NFATc1-4 is controlled by the phosphorylation status of the protein. Phosphorylated NFAT is predominantly localized in the cytosol. When Cn is active, NFAT is dephosphorylated, exposing two nuclear localization sequences (NLS) and inducing translocation to the nucleus, where it can activate transcription (Shaw *et al.*, 1995). Phosphorylation of NFAT by protein kinases such as GSK3β (Neal and Clipstone, 2001), DYRK1a (Kuhn *et al.*, 2009), p38-MAPK (Gomez del Arco *et al.*, 2000) and JNK (Chow *et al.*, 1997) leads to nuclear export of the protein (Hogan *et al.*, 2003). In response to ET-1 stimulation, multiple

NFAT isoforms have been shown to translocate to the nucleus of cultured cardiomyocytes (Kakita *et al.*, 2001; Kawamura *et al.*, 2004; Higazi *et al.*, 2009), with identity of the ET-1-regulated isoforms species and cell-type specific (Rinne *et al.*, 2010). Interestingly, nuclear import of Cn has also been observed in the cardiomyocyte, promoting sustained NFAT dephosphorylation and prolonging the NFAT-signal (Shibasaki *et al.*, 1996). Indeed, nuclear translocation of Cn is required for the response to ET-1 in NRVM (Hallhuber *et al.*, 2006; Higazi *et al.*, 2009).

Cn-NFAT activity within the cardiomyocyte is undoubtedly important for the hypertrophic effects of ET-1, and there is a wealth of further evidence that convincingly demonstrates the pathological role of Cn-NFAT activity within the heart. Failing human myocardium exhibits increased expression and activity of Cn and translocation of NFATc4 to the nuclear compartment (Lim and Molkenstein, 1999; Diedrichs *et al.*, 2004). These observations have been further substantiated by *in vitro* and *in vivo* studies. Overexpression of a constitutively-active mutant of Cn in the mouse heart induces severe left ventricular hypertrophy, cardiomyocyte dysfunction and heart failure (Molkenstein *et al.*, 1998). To further confirm these conclusions, other studies have probed the role of Cn-NFAT downstream of hypertrophic stimuli such as ET-1 *in vitro*. ET-1 increases Cn enzymatic activity in NRVM, inhibition of which prevents the response to hypertrophic stimulation (Zhu *et al.*, 2000). Moreover, multiple studies have verified that the deleterious effect of Cn on cardiac function is mediated through Cn-dependent regulation of one or more NFAT isoforms. Constitutively nuclear, constitutively active NFATc4 induces a phenotype of pathological left ventricular hypertrophy, confirming the damaging effect of increased NFAT-transcriptional activity *in vivo* (Molkenstein *et al.*, 1998). Moreover, cardio-specific deletion of either NFATc3 (Wilkins *et al.*, 2002) or NFATc2 (Bourajjaj *et al.*, 2008) impairs the hypertrophic response to increased Cn activity. Although these mouse models do not directly interrogate the role of ET-1-induced Cn-NFAT activity during hypertrophy, when combined with the evidence for ET-1-dependent NFAT translocation in isolated myocytes and the requirement for Cn in the hypertrophic response to ET-1, this pathway can be considered to be an important mediator of ET-1 induced hypertrophic responses.

Signalling to CaMKII, HDACs and MEF2 to induce hypertrophy

Although Cn-NFAT are strongly implicated in the hypertrophic response to ET-1, other studies have shown that ET-1-induced nuclear IICR elicits its pro-hypertrophic effect by engaging co-localized CaMKII and eliciting gene expression dependent upon the pro-hypertrophic transcription factor MEF2 (Wu *et al.*, 2006). Basally, MEF2 activity is restrained in the myocyte as a consequence of its association with class II histone deacetylases (HDACs 4,5,7 and 9), which negatively regulate transcription by deacetylating histone proteins, resulting in condensation of chromatin and impeding the access of transcriptional activators. In response to perinuclear IICR, CaMKII phosphorylates HDAC4 creating a

docking site for 14-3-3 proteins. Association of HDAC4 with 14-3-3 induces conformational changes that mask NLS and expose a nuclear export sequence, facilitating HDAC4 nuclear export and depressing MEF2-dependent transcriptional responses (Lu *et al.*, 2000; Zhang *et al.*, 2002; Wu *et al.*, 2006). ET-1 induces nuclear export of both HDAC4 and HDAC5 in rat ventricular myocytes (Wu *et al.*, 2006; Peng *et al.*, 2009), and ET-1 induced activation of MEF2-dependent transcription is likely to form a key component of the cellular response to the peptide. Deletion of MEF2D abrogates the hypertrophic response induced by hypertension-induced hypertrophy (Chang *et al.*, 2004; Kim *et al.*, 2008), whilst overexpression of MEF2A, C or D *in vivo* is sufficient to induce pathological cardiac remodelling (Kim *et al.*, 2008).

Recent reports indicate that the spatial pattern of ET-1-induced signalling is important in the regulation of HDAC phosphorylation. ET-1 activates CaMKII kinase activity, and this activity is necessary for the hypertrophic response to the peptide in NRVM (Zhu *et al.*, 2000). CaMKII itself is a key inducer of HDAC nuclear export, with cardiomyocytes predominantly expressing the CaMKII δ isoform, localized to either the nucleus (CaMKII δ _N) or cytosol (CaMKII δ _C) (Anderson, 2005). CaMKII δ _N is therefore present in the vicinity of its activating signal (ET-1-induced nuclear calcium transients) and its target (HDAC4). Although CaMKII directly targets HDAC4, it does not directly phosphorylate HDAC5. HDAC5 is rendered sensitive to CaMKII by forming oligomers with HDAC4 (Bucks *et al.*, 2008), indicating that ET-1-induced IICR could lead to nuclear export of both of the key MEF2 repressors. More recently, the novel calcium independent isoforms of PKC and the PKC target PKD have also been shown to phosphorylate HDAC5 and induce its nuclear export (Phan *et al.*, 2011). ET-1 activates both PKC and PKD in cardiomyocytes (Phan *et al.*, 2011); however, this is unlikely to form part of the mechanism whereby ET-1 depresses MEF2. ET-1-induced HDAC5 nuclear export is PKC-independent and although ET-1 activates subsarcolemmal PKD, it does not stimulate PKD translocation to the nucleus, preventing access of the kinase to its nuclear targets (Bossuyt *et al.*, 2011). Therefore, peri-nuclear IICR and the CaMKII activity it elicits are considered to be the key signals for ET-1-induced MEF2 depression.

ET-1 activation of PKC in hypertrophy

ET-1 activates the novel isoforms of PKC in cardiomyocytes. PKC isoform expression is species, tissue and cell-type-specific, with cardiomyocytes from murine species and humans expressing multiple differentially regulated PKC isoenzymes (Salazar *et al.*, 2007; Palaniyandi *et al.*, 2009). NRVM express PKCs α , δ , ϵ and η (Clerk *et al.*, 1995; Erdbrugger *et al.*, 1997; Markou *et al.*, 2006), whilst human cardiac tissue also expresses PKC β I and β II (Bowling *et al.*, 1999). In NRVM, ET-1 induces membrane association and activation of the DAG-sensitive, calcium-insensitive PKC δ and ϵ isoforms within 5 min of stimulation (Clerk *et al.*, 1994; Clerk and Sugden, 1997), and PKC ϵ is the major ET-1-activated isoform in AVM (Bogoyevitch *et al.*, 1993). The role of PKC isoenzymes in hypertrophy has been intensively investigated (Braz *et al.*, 2002; Vega *et al.*, 2004; Vijayan *et al.*, 2004). This has

lead to the identification of many downstream targets of PKC relevant to hypertrophy in cardiomyocytes, such as the small G-protein Ras (Montessuit and Thorburn, 1999), mTOR, ribosomal S6 kinase (Moschella *et al.*, 2007), GSK3 β (Zhai *et al.*, 2007), GATA4 (Wang *et al.*, 2005) and PKD (Vega *et al.*, 2004). Although PKC regulates multiple targets, activation of Ras and downstream MAPK cascades by the DAG-sensitive isoforms of PKC is considered to have a predominant role in cardiac remodelling (Braz *et al.*, 2002).

MAPK signalling pathways in the hypertrophic response

Upon ET-1 binding to the ET_A receptor, the classical small G protein Ras is converted into an active, GTP-bound form. Multiple mechanisms have been suggested to explain this effect, relying on the activity of PKC as described above and/or ET-1-induced production of intracellular reactive oxygen species (ROS) (Sugden and Clerk, 2006). Clinically, activating mutations in Ras and its target Raf result in Costello and Noonan syndromes respectively, both of which induce cardiomyopathy amongst other abnormalities (Lin *et al.*, 2011; Wu *et al.*, 2011). Induction of hypertrophy by activated Ras and Raf is ERK1/2 dependent, suggesting that this kinase is responsible for the hypertrophic response to Ras and Raf activation (Harris *et al.*, 2004; Wu *et al.*, 2011). However, although it has been intensively investigated, the precise role and function of activated ERK1/2 in the heart remains controversial (Bueno and Molkentin, 2002; Kehat and Molkentin, 2010). It has been appreciated for many years that ERK1/2 is activated in NRVM in response to hypertrophic agonists including ET-1 (Bogoyevitch *et al.*, 1994). Moreover, inhibition of ERK1/2 activation using either pharmacological inhibitors of its activating kinases MEK1/2 or adenoviral-mediated overexpression of dominant-negative MEK prevents the hypertrophic response to ET-1 in NRVM (Ueyama *et al.*, 2000; Yue *et al.*, 2000). Indeed, microarray studies demonstrated the reliance of ET-1-induced hypertrophic gene expression on ERK1/2 activity (Marshall *et al.*, 2010). However, these observations have been called into question following *in vivo* studies of transgenic mouse models. Cardiac-specific overexpression of constitutively-active MEK1 induces ERK1/2 phosphorylation and compensated concentric hypertrophy (Bueno *et al.*, 2000). Therefore, activation of ERK1/2 above the baseline level, as occurs *in vitro* in response to ET-1, can induce cardiac remodelling. However, *in vivo* loss-of-function studies of ERK1/2 have demonstrated that under certain conditions the hypertrophic response is preserved, calling into question the necessity of these kinases during pathology. Echoing the current thinking regarding calcium-regulated signalling, interesting observations suggest that spatial and contextual aspects of ERK1/2 activation may govern whether it exhibits pro-hypertrophic activity. Interestingly, in vascular smooth muscle cells and fibroblasts, ET-1 induces nuclear translocation of ERK1/2 (Lenormand *et al.*, 1993; El-Mowafy and White, 1999) where it can phosphorylate targets involved in regulation of gene transcription. These include transcription factors (e.g. GATA4 and Elk), nuclear kinases (e.g. MSK1) and chromatin modify-

ing enzymes (e.g. the p300 histone acetylase). In cardiomyocytes, a novel phosphorylated form of ERK1/2 (p-Thr188-ERK1/2) accumulates within the nucleus over the duration of the hypertrophic response *in vivo*, an effect that could also be simulated *in vitro* by agonists of G α_q -coupled receptors (Lorenz *et al.*, 2009). Hence, it is conceivable that ERK1/2 activity becomes more important in the later stages of hypertrophy, once sufficient nuclear accumulation has occurred. Furthermore, the basal and stimulus-induced levels of ERK1/2 activity may have different functional effects within the myocyte (Kehat *et al.*, 2011). In the context of cellular stimulation with agonists such as ET-1, mechanisms may be activated that permit ERK1/2 to perform functions other than those active under non-stimulated conditions, changing the cellular response to the kinase. Discriminating whether, when and how ERK1/2 regulates hypertrophy is an interesting topic of future research.

Although the requirement for ERK1/2 during remodelling remains controversial, many of the targets of the kinase have defined pro-hypertrophic roles. Genome-wide studies of myocyte gene expression profiles have shown that ET-1 induces distinct waves of gene expression, with the earlier phases of the response being largely ERK1/2 dependent (Cullingford *et al.*, 2008; Marshall *et al.*, 2010). Demonstrating the importance of these acute events, inhibiting activity of the activator protein-1 (AP1) complex of the immediate-early genes c-fos and c-jun prevents ET-1-induced hypertrophic remodelling (Omura *et al.*, 2002). Other transcription factors also exhibit ET-1-induced ERK1/2-dependent activity. For example, GATA4 shows ET-1-dependent DNA-binding activity in response to increased myocardial stretch (Hautala *et al.*, 2001; 2002; Liang *et al.*, 2001) and is regulated by ERK1/2-dependent phosphorylation (Liang *et al.*, 2001). Expression of GATA4 harbouring phospho-mimetic activating mutations is pro-hypertrophic both *in vitro* and *in vivo*, whilst replacing endogenous GATA4 with a mutant that cannot be phosphorylated on its activating sites impedes hypertrophic responses (van Berlo *et al.*, 2011). Interestingly, GATA4 can form higher order complexes with other pro-hypertrophic transcription factors such as NFAT and AP1, providing a mechanism whereby pro-hypertrophic signalling pathways can be integrated (Sanna *et al.*, 2005).

Are JNK and p38-MAPK important for ET-1-induced remodelling?

Early studies in NRVM demonstrated that both cellular osmotic stress and ET-1 induce phosphorylation and activation of JNK1/2. However, the response to stress-associated stimuli was much greater than the response to ET_A receptor activation. Subsequently, the role of JNK1/2 in hypertrophy has been extensively examined. Overexpression of dominant-negative MKK4, the kinase upstream of JNK1/2, attenuates the hypertrophic response to ET-1 stimulation of NRVM (Choukroun *et al.*, 1998). These and other data suggest that JNK1/2 mediates a pro-hypertrophic effect *in vitro*. However, this observation has been questioned by other studies showing that activation of JNK1/2 *in vitro* inhibits the hypertrophic response to ET-1 (Nemoto *et al.*, 1998). To attempt to

address the conflicting data sets, *in vivo* studies have been performed. Currently, the prevailing consensus suggests that although ectopic activation of JNK1/2 can induce cardiac remodelling and eventual heart failure (Choukroun *et al.*, 1999), these changes occur in the absence of left ventricular hypertrophy. Instead, myocardial stiffening has been observed as a consequence of extracellular matrix remodelling (Petrich *et al.*, 2004). Intriguingly JNK1^{-/-} knock-out mice exhibited a hastened early deterioration in cardiac performance during hypertension-induced hypertrophy, suggesting that the basal level of activity of this isoform may be important for maintaining cardiac function (Tachibana *et al.*, 2006). The cardio-protective actions of JNK have been attributed to JNK-dependent phosphorylation and subsequent nuclear export of the highly pro-hypertrophic transcription factor NFAT (Chow *et al.*, 1997) and/or JNK1/2-dependent positive regulation of the anti-hypertrophic protein JunD. The importance of JNK activity within the hypertrophic response to ET-1 is likely to be governed by the balance of stimuli within the ET-1-regulated signalling network.

Similarly to JNK, the exact role of p38-MAPK within the myocardium has proven to be a matter for debate, with data generated *in vitro* and *in vivo* producing somewhat conflicting conclusions. Both ET-1 and cellular stress induce p38-MAPK phosphorylation and activation. However, the kinase has been demonstrated to either be necessary or dispensable for the ET-1-induced hypertrophic response. Furthermore, the different isoforms of p38-MAPK may have variable pathological roles, with p38-MAPK α implicated in the induction of apoptosis whilst p38-MAPK β is involved in hypertrophy. The weight of evidence suggests that p38-MAPK activity is not required for the initial development of left ventricular hypertrophy (Liao *et al.*, 2001; Braz *et al.*, 2003; Zhang *et al.*, 2003; Nishida *et al.*, 2004). Indeed, akin to the action of JNK1/2, p38-MAPK isoforms may be anti-hypertrophic as a consequence of their NFAT-kinase activity during the early hypertrophic response (Gomez del Arco *et al.*, 2000; Braz *et al.*, 2003). In the latter stages of cardiac decompensation and heart failure, pathological functions for p38-MAPK have been described. The kinase promotes ventricular dilation, stiffening and fibrosis thereby impairing contractility (Liao *et al.*, 2001; Zhang *et al.*, 2003). Consequently, the effect of ET-1 stimulated p38-MAPK activity may not be important for the initial response to the peptide, but become more significant over time. A possible reason for this is that the remodelled physiology and signalling pathways of the hypertrophic heart creates a different profile of substrates or cellular context for the action of p38-MAPK.

ET-1 signalling – a question of location and timing?

When considered in their entirety, the importance of ET-1-induced signalling pathways within the hypertrophic response appears to be determined by two governing rules. First, as described above, as a consequence of the remodelling of gene expression that occurs during hypertrophy, the necessity of the various ET-1-regulated mediators for the induction of pathology may be altered over time. Secondly, subcellular

localization of ET-1-dependent signals is a critical determinant of their pro-hypertrophic role. This is elegantly demonstrated by the specific pro-hypertrophic effect of peri-nuclear release of calcium in response to IP₃RII activation. Interestingly, the ET_A receptor itself may also be subject to regulated localization, a mechanism that could be important for its cellular effects. The localization of GPCRs has been well studied and several GPCRs are localized at the nuclear membrane of cardiac myocytes and other cells types (Tadevosyan *et al.*, 2010; Vaniotis *et al.*, 2011a; 2011b; Wright *et al.*, 2012). Although ET_A receptors are largely found within the sarcolemma, several studies have demonstrated functional ET_A receptor and ET_B receptor at the nuclear envelope of ventricular cardiomyocytes (Boivin *et al.*, 2003; Bkaily *et al.*, 2011). This may result from translocation of ET receptors from the cell surface or by *de novo* receptor synthesis. In order for nuclear receptors to be functional, a source of intracellular ligand must exist. It has been hypothesized that ET-1 produced intracellularly in the myocyte can signal in an intracrine manner and that ET-1 is taken into the cell from an extracellular source to act on nuclear ET receptors (Boivin *et al.*, 2003; Tadevosyan *et al.*, 2012). Activation of nuclear ET_A receptor has been postulated to stimulate similar downstream pathways as sarcolemmal ET_AR, such as PKC activation and IP₃ generation, thus potentially activating hypertrophic gene expression and regulating calcium homeostasis (Bkaily *et al.*, 2011). As ET-1 production is increased in hypertrophic and failing myocytes, under hypertrophic conditions, there would be a larger stimulus to ET receptors located within the cell. Furthermore, if present in the nucleus specifically, the ET_A receptor and the signalling pathways it initiates would be clustered in close proximity of their cellular effectors, facilitating ET-1-dependent cellular responses. Further studies to substantiate the localization of ET_A receptors within the nucleus and their significance and relevance to the hypertrophic response are required to allow inclusion of subcellular ET-1 signalling within the accepted models of ET-1 action.

Non-myocyte effects of ET in the heart

ET-1 signalling within the heart regulates a wide spectrum of cardiovascular processes, ranging from development through to life-threatening cardiovascular diseases. In this review, we have focused upon ET signalling in cardiac myocytes. By inducing the activity of a complex web of signalling pathways, this important peptide can directly influence myocyte survival, contractility and remodelling. However, the heart is composed of multiple cell types and despite providing the greater part of cardiac mass, myocytes account for only ~20% of the total number of cells in humans (Rubart and Field, 2006). The non-myocyte population is comprised of endothelial cells, smooth muscle cells and fibroblasts (Nag, 1980; Banerjee *et al.*, 2007), all of which are responsive to ET-1 (Kedzierski and Yanagisawa, 2001). Cardiac endothelial and smooth muscle cells form the myocardial vasculature. ET-1 interacts with these cells to regulate vascular tone, influencing myocardial blood supply and metabolic activity (Wang *et al.*, 1998). Cardiac fibroblasts are essential for secretion of extracellular matrix and for cardiac repair (Biernacka and

Frangogiannis, 2011). In response to elevated levels of ET-1, cardiac fibroblasts proliferate, differentiate into myofibroblasts and increase their secretion of matrix proteins (Shi-Wen *et al.*, 2004a; 2004b). These fibroblast responses underlie scar formation following cardiac damage and the myocardium-wide fibrosis associated with cardiac hypertrophy, where the increased abundance of cardiac fibroblasts replaces the myocytes lost as a result of pathology (Swynghedauw, 1999). The reactive response of the fibroblast is essential for the maintenance of cardiac function during disease, without which the integrity of the heart is compromised (Shimazaki *et al.*, 2008). However, fibroblast infiltration alters the electrophysiological properties of the myocardium, affecting both action potential propagation and allowing arrhythmic events at the cellular level to propagate (Mayyas *et al.*, 2010; Karagueuzian, 2011). Cardiac fibrosis also decreases elasticity of the heart, stiffening the myocardial wall and impeding cardiac output (Lakatta and Levy, 2003a). Extensive fibrosis of the myocardium is not only a characteristic of pathology but is also a prominent feature of the aged, senescent heart and is common with pathological situations, fibrosis of the aged heart arises due to hypertrophy and myocyte loss (Lakatta and Levy, 2003a; Biernacka and Frangogiannis, 2011). When combined with the decreased relaxation rate of the aged heart, these effects are manifest in diastolic dysfunction, impacting exercise tolerance, quality of life and contributing to future heart failure (Lakatta and Levy, 2003a). As ET-1 increases in concentration with age (Maeda *et al.*, 2003) and induces fibrosis (Shi-Wen *et al.*, 2004a), the importance of the ET-1 system for the decline in myocardial function associated with age should be considered paramount.

Role of ET-1 in PAH and right ventricular hypertrophy

The compelling evidence linking the ET-1 system to cardiovascular pathologies has prompted its targeting for therapeutic benefit. Currently, the combined ET_A-ET_B receptor antagonist bosentan and the selective ET_A receptor antagonist ambrisentan are licensed and successfully used for the treatment of pulmonary arterial hypertension (PAH) (Kirkby *et al.*, 2008), a condition which induces right ventricular hypertrophy, right heart failure and eventually death (Gomberg-Maitland *et al.*, 2011). PAH increases pulmonary arterial pressure and vascular resistance, stretching and increasing the load on the right ventricle. Under these conditions, the type 1 angiotensin receptor is activated in response to myocardial stretch and stimulates ROS formation. Subsequently, increased intracellular ROS indirectly triggers the release of ET-1. ET-1-induced increases in intracellular calcium stimulate NFAT and CaMKII, culminating in hypertrophic remodelling (Cingolani *et al.*, 2010). Interestingly, during PAH, prohypertrophic GATA4 is also active within the right ventricle (Park *et al.*, 2010), suggesting that multiple ET-1 regulated signalling pathways may be important in this condition. Significantly, ET_A receptor blockade prevents right ventricular remodelling in an animal model (Jasmin *et al.*, 2003), demonstrating the importance of the link between PAH, right ventricular hypertrophy and ET-1. These effects may be

responsible for the therapeutic benefit of ET_A receptor antagonists for the treatment of PAH.

Why have ET receptor antagonists failed in the treatment of left ventricular hypertrophy and congestive heart failure and where may they provide benefit?

Despite the myriad of lines of evidence from both animal models and humans for a role of ET-1 and ET-1-dependent signalling pathways in left ventricular hypertrophy and congestive heart failure (CHF), it is disappointing to note that ET-1 receptor antagonists have not yet proven efficacious for the treatment of CHF (Kirkby *et al.*, 2008). Although initial studies were promising (Sutsch *et al.*, 1998), clinical trials of the combined ET_A-ET_B receptor antagonist bosentan for the treatment of CHF were halted owing to problems with liver toxicity. Furthermore, later studies were unable to demonstrate a reduction in the mortality or symptoms of patients suffering from CHF when a lower dose of bosentan was administered (Kalra *et al.*, 2002; Kelland and Webb, 2006). A number of explanations for the failure of this approach have been proposed. Blockade of the ET_B receptor impairs ET-1 clearance, increasing plasma ET-1 levels and allowing the effects of ET_A receptor antagonism to be overcome (Cowburn *et al.*, 2005). Furthermore, ET_B receptor inhibition leads to systemic vasoconstriction, a deleterious response in patients with cardiovascular conditions (Cowburn *et al.*, 2005). To avoid the complicating effects of ET_B receptor inhibition, studies aiming to selectively block the ET_A receptor have been carried out (Anand *et al.*, 2004; Cowburn *et al.*, 2005; Leslie *et al.*, 2005). Although cardiac output was acutely improved, no long-term benefit of this treatment regimen was observed (Anand *et al.*, 2004). However, these studies may have utilized a dose of drug at which antagonism of the ET_B receptor cannot be completely excluded (Kelland and Webb, 2007). In view of such limitations, it would be informative if trials were performed using ET_A receptor antagonists at a dose that more selectively targets the ET_A receptor. In addition, the results of many of the clinical trials performed to date have not been fully published and analysed, meaning that a thorough interpretation of the data is lacking (Kelland and Webb, 2007). Further progress will only be made by a more detailed analysis of these trials. For example, there may be patient subpopulations where ET receptor inhibition is effective. It will also be important to gain a better understanding of the interaction of the ET-1 system with patient lifestyle and age. Indeed, ET-1 levels are elevated in aged and obese populations, which may suggest that ET receptor antagonists would provide greater benefit in these individuals. Interestingly, improved lifestyle benefits these populations, with weight loss and exercise reducing ET-1 levels in the obese and aged respectively (Maeda *et al.*, 2003; 2006). Furthermore, our increased understanding of ET-1 biology and ET-1-induced cellular responses may henceforth guide more intelligent and targeted drug design. In view of the disparate effects of ET_A and ET_B receptor inhibition in humans, it will be

important to consider the influence of dimers and heterodimers of the ET receptor subtypes upon downstream signalling and pharmacology (Evans and Walker, 2008a; 2008b). More knowledge regarding the persistence, localization and integration of these ET-1-induced responses is also required. Agents that allow selective control of the activation of ET_A and ET_B receptors and/or the individual limbs of the signalling pathways they activate downstream would be particularly informative. The development of biased agonists/antagonists akin to those for the angiotensin receptor would allow for the specific targeting of key signalling pathways (Watts, 2010) and perhaps provide a window of opportunity to manipulate ET-1-induced signalling for therapeutic benefit.

Cardiac failure is the dominant cause of mortality in the over 65 population. This population is likely to double in number in the next 25 years; hence, future therapies will need to be developed to tackle the increasing burden of cardiac disease. The prominent role that ET-1 performs in the pathological alterations in physiology and function of the multiple cell types of the heart, in particular during ageing, identify it as a key target for further development of therapeutic strategies.

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Conflict of interest

None.

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